MicroRNA-141 confers resistance to cisplatin-induced apoptosis by targeting YAP1 in human esophageal squamous cell carcinoma

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MicroRNAs (miRNAs) are endogenous non-coding RNAs that function as negative regulators of gene expression. Alterations in miRNA expression have been shown to affect tumor growth and response to chemotherapy. In this study, we explored the possible role of miRNAs in cisplatin resistance in esophageal squamous cell carcinoma (ESCC). First we assessed the sensitivity of nine human ESCC cell lines (KYSE series) to cisplatin using an in vitro cell viability assay, and then we compared the miRNA profiles of the cisplatin-sensitive and -resistant cell lines by miRNA microarray analysis. The two groups showed markedly different miRNA expression profiles, and 10 miRNAs were found to be regulated differentially between the two groups. When miR-141, which was the most highly expressed miRNA in the cisplatin-resistant cell lines, was expressed ectopically in the cisplatin-sensitive cell lines, cell viability after cisplatin treatment was increased significantly. Furthermore, we found that miR-141 directly targeted the 3'-untranslated region of YAP1, which is known to have a crucial role in apoptosis induced by DNA-damaging agents, and thus downregulated YAP1 expression. Our study highlights an important regulatory role for miR-141 in the development of cisplatin resistance in ESCC.

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USA) supplemented with 10% fetal bovine serum. Cells were cultured at 37°C with 5% CO2.

**In vitro cell viability assay**

KYSE cell lines were seeded in 96-well plates and incubated for 24 h. The medium was then removed and replaced with fresh medium that contained cisplatin (Calbiochem, San Diego, CA, USA) or Dimethyl sulfoxide (DMSO) (vehicle control) and the cells were incubated for a further 48 h. Cell viability was measured by the WST-1 assay using the 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-1) assay.

**miRNA microarray analysis**

Total RNA was isolated from the KYSE cell lines with IsoGen lysis buffer (Nippon Gene, Toyama, Japan) followed by precipitation with isopropanol, and the size of the miRNA fractions was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The miRNAs were then labeled with Hybrid 75 Cy3/Cy5 microRNA Array Labeling Kit (Exiqon, Woburn, MA, USA) and hybridized with a Human miRNA Oligo chip (Toraq, Tokyo, Japan). Arrays were scanned using a ProScanArray laser scanning system (Perkin-Elmer, Waltham, MA, USA), and processed and analyzed with GenePix Pro 4.0 software (Axon Instruments, Sunnyvale, CA, USA). The GEO database accession code of the miRNA microarray data is GSE25464.

**TagMan RT-PCR for miRNA quantification**

Expression levels of mature miRNAs were analyzed by real-time PCR using the TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA, USA). Reactions were performed using an Applied Biosystems 7300 instrument with an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

**In vitro drug sensitivity assay**

KYSE960 cells (1.7 × 104 per well) were seeded in 96-well plates and transfected with either Pre-miR miR-141 precursor or Pre-miR miRNA Precursor-Negative Control #1 (AM17110) (Ambion, Austin, TX, USA) using the HiPerfect Transfection Reagent (Qiagen, Valencia, CA, USA). The cells were incubated with cisplatin (30 µM, 48 h) or cisplatin (30 µM, 48 h) and collected for analysis after 0, 24, 48 and 72 h. An equal volume of 0.4% trypan blue dye exclusion assay

At 24 h after transfection, cells with cisplatin (30 µM) or DMSO were collected for analysis. For each 24-well plate with 1 µg of the firefly luciferase reporter vector and 100 ng of pRL-TK (a control vector that contains Renilla luciferase; Promega) as well as with 5 pmol of miR-141 or a control precursor (Ambion) or with 25 pmol of miR-141 or a control inhibitor (Dharmacon, Lafayette, CO, USA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase activity was measured at 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega). For each well, firefly luciferase activity was normalized to Renilla luciferase activity.

**Apoptosis assay**

At 24 h after transfection, cells were treated with cisplatin (30 µM) or DMSO for a further 48 h and then collected for analysis. Apoptosis was assayed using a FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Apoptotic cells were indicated by high iodide fluorescence and were quantitated by flow cytometry.

**Statistical analysis**

Statistical significance was assessed using an unpaired Student's t-test. P < 0.05 was considered to be statistically significant. Data were expressed as mean ± s.e.m.

**RESULTS**

Cisplatin-sensitive and -resistant human ESCC cell lines

To classify the ESCC cell lines (KYSE series) into cisplatin-sensitive and -resistant cell lines, cell viability of nine KYSE cell lines in the presence of cisplatin was analyzed using the WST-1 assay (Figure 1). Cisplatin treatment (66.7 µM, 48 h) resulted in a decrease in cell viability of 0–80%, as compared with cells treated with the vehicle DMSO. Among the cell lines examined, KYSE980 and KYSE960 showed the highest sensitivity to cisplatin treatment (~20% cell viability), whereas KYSE450 and KYSE520 showed the lowest sensitivity to cisplatin treatment (~80–100% cell viability). We consequently designated the cell lines KYSE980 and KYSE960 as cisplatin sensitive and KYSE450 and KYSE520 cell lines as cisplatin resistant.
miR-141 is highly expressed in cisplatin-resistant ESCC cell lines

We then compared the expression of miRNAs in cisplatin-sensitive and -resistant KYSE cell lines using miRNA microarray analysis. Total RNA was isolated from the KYSE cell lines and hybridized to a custom miRNA microarray platform that contained 849 miRNAs. The global miRNA expression analyses (hierarchical clustering and principal component analysis) showed that the expression profiles of the miRNAs differed between the cisplatin-sensitive and -resistant cell lines (Figure 2), and expression levels of 45 miRNAs were changed by more than fourfold in cisplatin-sensitive cell lines as compared with cisplatin-resistant cell lines (Supplementary Table 1). Subsequently, the expression levels of the 10 miRNAs that were selected according to the miRNA microarray data and literature search were validated by quantitative reverse transcription-PCR. This confirmed that miR-141, miR-21, miR-19b, miR-200a, miR-19a, miR-27a, miR-20a, and miR-20b were expressed at significantly higher levels in the cisplatin-resistant lines, and miR-205 and miR-224 at significantly lower levels than in the cisplatin-sensitive cell lines (P < 0.05) (Table 1). Notably, miR-141 was upregulated the most in the cisplatin-resistant lines as in contrast with the cisplatin-sensitive lines (87-fold, P = 0.01) (Figure 3).

Figure 1 Designation of cisplatin-sensitive and -resistant human ESCC cell lines. The WST-1 activity of cells treated with DMSO was designated as 1 and the relative WST-1 activity is shown.

Figure 2 Global miRNA expression analysis of ESCC cell lines. Hierarchical clustering (a) and principal component analysis (b) of global miRNA expression in ESCC cell lines. These analyses reveal different miRNA expression profiles between cisplatin-sensitive and -resistant cell lines.

Table 1 List of miRNAs that were expressed differentially in cisplatin-sensitive and cisplatin-resistant cell lines

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Cisplatin-sensitive cell lines (mean ± s.e.m.)</th>
<th>Cisplatin-resistant cell lines (mean ± s.e.m.)</th>
<th>Fold change</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-141</td>
<td>189.67 ± 24.95</td>
<td>16587.80 ± 4145.71</td>
<td>87.45</td>
<td>0.0108</td>
</tr>
<tr>
<td>miR-21</td>
<td>64.57 ± 10.62</td>
<td>3157.66 ± 511.71</td>
<td>49.90</td>
<td>0.0018</td>
</tr>
<tr>
<td>miR-19b</td>
<td>1717.65 ± 526.16</td>
<td>59809.56 ± 5297.46</td>
<td>34.82</td>
<td>0.0001</td>
</tr>
<tr>
<td>miR-200a</td>
<td>161.26 ± 45.91</td>
<td>2024.54 ± 105.95</td>
<td>12.55</td>
<td>0.0001</td>
</tr>
<tr>
<td>miR-19a</td>
<td>383.92 ± 95.89</td>
<td>27437.61 ± 1074.00</td>
<td>71.47</td>
<td>0.0001</td>
</tr>
<tr>
<td>miR-27a</td>
<td>84.33 ± 19.15</td>
<td>2313.07 ± 278.58</td>
<td>27.43</td>
<td>0.0005</td>
</tr>
<tr>
<td>miR-20a</td>
<td>1620.13 ± 435.78</td>
<td>79664.13 ± 15638.85</td>
<td>49.17</td>
<td>0.0041</td>
</tr>
<tr>
<td>miR-20b</td>
<td>57.90 ± 18.89</td>
<td>1063.99 ± 225.53</td>
<td>18.38</td>
<td>0.0065</td>
</tr>
<tr>
<td>miR-205</td>
<td>22770.74 ± 3752.22</td>
<td>2360.90 ± 623.31</td>
<td>0.10</td>
<td>0.0026</td>
</tr>
<tr>
<td>miR-224</td>
<td>1680.99 ± 131.29</td>
<td>444.79 ± 283.08</td>
<td>0.26</td>
<td>0.0054</td>
</tr>
</tbody>
</table>

Abbreviation: miRNA, microRNA.
Ectopic expression of miR-141 confers cisplatin resistance in cisplatin-sensitive cell lines

To investigate whether miR-141 is involved directly in the development of cisplatin resistance, we examined the effects of miR-141 on cisplatin sensitivity. We expressed the miR-141 precursor ectopically in the cisplatin-sensitive cell lines, because they express relatively low levels of endogenous miR-141, and examines whether miR-141 expression rendered the cells resistant to cisplatin-induced cell death. Following transfection of the miR-141 precursor, the cells were collected and ectopic expression of miR-141 was confirmed by quantitative reverse transcription-PCR (Figure 4a). Cells transfected with the control precursor were used as controls. The results of the WST-1 assay showed that the KYSE960 cells that had been transfected with the miR-141 precursor exhibited a markedly reduced sensitivity to varying concentrations of cisplatin (7.5, 15, 30, 60 or 120 \( \mu \)M) (Figure 4b).

Similar findings were obtained with KYSE890 cells (data not shown). Moreover, the trypan blue dye exclusion assay revealed that the proportion of KYSE960 cells, which remained viable after treatment with cisplatin (30 \( \mu \)M), was significantly \((P<0.05)\) elevated at each time point in cells that overexpressed miR-141: 18, 68 and 48% at 24, 48 and 72 h after cisplatin treatment, respectively (Figure 4c). These results indicated that ectopic expression of miR-141 could confer cisplatin resistance in KYSE cell lines by enhancing their growth and viability.

miR-141 represses YAP1 expression post transcriptionally

In an effort to elucidate the mechanism of induction of cisplatin resistance by miR-141, we searched for potential target(s) of miR-141 using the TargetScan database (http://www.targetscan.org/). Among the predicted 429 candidate genes, we studied the functional role of human Yes-Associated Protein (YAPI) (NM_006106) further, because it has been reported to be a cisplatin-induced apoptosis-related gene. First, we investigated the effects of transfection of the miR-141 precursor on YAPI expression in cisplatin-sensitive KYSE cell lines. The results indicated that ectopic expression of miR-141 could confer cisplatin resistance in KYSE cell lines by enhancing their growth and viability.
In contrast, when the wild-type reporter was co-transfected with the miR-141 inhibitor, the relative luciferase activity of the reporter was significantly \( P < 0.05 \) enhanced (Figure 5d). These results show that miR-141 interacts directly with the predicted target sequence in \( YAP1 \).

miR-141 exerts an anti-apoptotic effect that confers cisplatin resistance in ESCC cell lines

Given that one of the target genes of miR-141 is \( YAP1 \), which is a transcriptional factor that promotes the expression of proapoptotic genes during apoptosis induced by DNA-damaging agents, we explored the regulatory mechanism by which miR-141 inhibits cisplatin-mediated apoptosis. The Annexin V/propidium iodide assay showed that apoptosis of the cisplatin-sensitive ESCC cell lines (KYSE960 and KYSE890) in response to cisplatin was enhanced markedly compared with that of the cisplatin-resistant cells (data not shown). In both KYSE960 and KYSE890 cells, transfection of the miR-141 precursor, but not the control precursor, significantly decreased the percentage of cisplatin-induced apoptotic cells (Figure 6). Taken collectively, these results show that the anti-apoptotic effect of miR-141, perhaps through inhibition of \( YAP1 \),
might explain how miR-141 confers cisplatin resistance in ESCC cell lines.

**DISCUSSION**

In the present study, we explored the possible role of miRNAs in cisplatin resistance in ESCC. By comparing the expression of miRNAs in cisplatin-sensitive and -resistant KYSE series, we found 10 miRNAs that were expressed differentially between these lines. Among them were some miRNAs, such as miR-21, miR-20b, miR-205, miR-224, miR-20a and miR-141, which are known to be associated with cancer. miR-21 has been reported to be ubiquitously overexpressed in diverse tumors, including both esophageal adenocarcinoma and ESCC, and it regulates proliferation and invasion in ESCC. In addition, inhibition of miR-21 has been shown to increase the sensitivity of NC160 and cholangiocarcinoma cell lines to chemotherapeutic agents. miR-20b is highly overexpressed in ESCC and gastric cancer, and its high-expression level is associated with a lower probability of survival. The expression of miR-205 is highly specific for squamous epithelium, and it has been shown to be downregulated in both esophageal adenocarcinoma and ESCC. miR-205 has also been found to function as an oncosuppressor in breast cancer and to improve responsiveness to tyrosine kinase inhibitor therapies. Furthermore, miR-224, miR-27a and miR-200a have also been associated with hepatocarcinoma, ESCC and ovarian cancer, respectively. miR-141 is associated with various types of cancer. Given that miR-141 was found to be either upregulated (ovarian and colorectal cancers) or downregulated (prostate, hepatocarcinoma and renal cell carcinoma) in various cancers, it appears that miR-141 might have different roles, as either an oncosuppressor or a tumor-suppressor gene, in different cancer types of cancer. Therefore, most of the differentially expressed miRNAs identified in this study by comparing miRNA expression in cisplatin-sensitive and -resistant human ESCC cell lines appear to show some involvement in cancer; however, none of these miRNAs has previously been found to be associated with the development of cisplatin resistance.

Our study further showed that miR-141, which was the most upregulated miRNA in cisplatin-resistant ESCC cell lines, conferred cisplatin resistance in ESCC. Upon ectopic expression of miR-141, the viability of the cisplatin-sensitive cell lines after cisplatin treatment was elevated significantly. This effect was due to the inhibition of cisplatin-induced apoptosis by miR-141, which indicated that miR-141 is an anti-apoptotic factor. Furthermore, we found that miR-141 negatively regulates the expression of YAP1. YAP1 is a well-documented pro-apoptotic transcriptional factor, and inhibition of its expression greatly reduces cisplatin-induced apoptosis. Given that the results of our present study showed that miR-141 targets YAP1 and negatively regulates the expression of YAP1, it is likely that miR-141 exerts its anti-apoptotic effect, at least in part, through repressing YAP1 expression.

In summary, our study provides the first evidence that miR-141 has a key role in cisplatin resistance in ESCC, because of its anti-apoptotic properties. Our study highlights the potentially important role of miRNAs in the development of drug resistance, and suggests that miRNAs might serve as biomarkers for response to chemotherapy.

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